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PRINCIPAL INVESTIGATOR: Annalisa D'Andrea, PhD

CONTRACTING ORGANIZATION: SRI International, Menlo Park,CA,94025

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Annalisa D'Andrea

e-mail:Annalisa.dandrea@sri.com

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SRI International, 333
Ravenswood Avenue,
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14. ABSTRACT

The goal of this project is to develop miR-326 small molecule inhibitors (SMIs) for the treatment of Multiple Sclerosis (MS). Our global approach consists of creating stable transfectants with a luciferase reporter gene and 3' target sequence for miR-326. The addition of hsa-miR-326 (delivered either by lentivirus or cotransfection) results in reduced fluorescence, that can be restored by the addition of inhibitors of miR-326. Our efforts to develop a screening method for miR-326 were not successful therefore we were not able to screen compounds. Additionally, experiments aimed to reproduce data showing an association of miR-326 with Th17 cells failed to support that concept. We did not observe any up-regulation of miR326 in Th17 over other T cells.

15. SUBJECT TERMS
Multiple Sclerosis, miR-326, Luciferase, miR-21, Th17

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INTRODUCTION

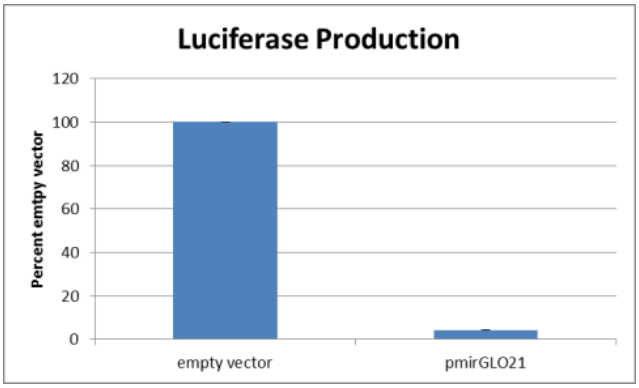
The goal of this project is to develop miR-326 small molecule inhibitors (SMIs) for the treatment of Multiple Sclerosis (MS).

BODY

MicroRNAs are small non-coding RNAs roughly 23 nucleotides in length that function as gene silencers. MicroRNAs are able to partially silence genes by interfering with translation or the stability of target mRNAs. They exert profound effects in a variety of disease models, including cancer and autoimmune disorders, and modulate many basic cellular processes [1]. One microRNA, miR326, has been reported to affect development of Th17 cells, a pathogenic T cell subset associated with multiple sclerosis (MS) [2]. Moreover, this microRNA is elevated in the brains [3] and peripheral blood mononuclear cells (PBMCs) of MS patients [4]. Previous publications have also shown that microRNAs can be inhibited by small molecules [5], suggesting that small molecule inhibitors (SMIs) of miR326 could be used therapeutically to limit inflammation associated with MS and reduce disease severity. The goal of this project was to develop miR326 small molecule inhibitors for the treatment of Multiple Sclerosis.

In order to screen SMIs, we proposed to develop a bioassay to determine the efficacy and specificity of SMIs. We first created stably and transiently transfected cell lines that overexpress miR326. In addition, to allow measurement of miR326 activity, we introduced a complementary miR326 binding sequence along with a luciferase reporter construct into the cells. This reporter construct allows for both measurement of the transfection efficiency by Renilla luciferase, but also detect the degradation of complementary mRNA for the microRNA of interest by measuring levels of Firefly luciferase. The addition of hsa-miR326 (delivered either by lentivirus or cotransfection) should result in reduced Firefly luminescence, with no change in Renilla luminescence. The addition of putative inhibitors will restore the Firefly luciferase-dependent production. As reported in the first year progress report, we put considerable effort into troubleshooting unforeseen problems with transfection as well as in establishing stable cell lines expressing constructs of interest.

We selected HeLa cells because they express high levels of miR21 allowing us to use miR21 as an endogenous positive control in addition to generating transfectants that also express miR-326. By comparing HeLa cells with or without miR326, this system provides an ideal control for screening for SMIs that are miR326 specific or can interfere with other miRs. As a control, we transfected either pmirGLO Dual-Luciferase miRNA Target Expression Vector with no miRNA binding site (empty vector) or pmirGLO containing the miR21 binding sequence. In addition, we transfected the cells with or without the miR326 overexpression construct. As expected, we observed robust luciferase activity with the empty vector, while there was little activity using the pmirGLO21 plasmid because the endogenous miR21 was able to bind the luciferase mRNA and suppress activity (Figure 1).



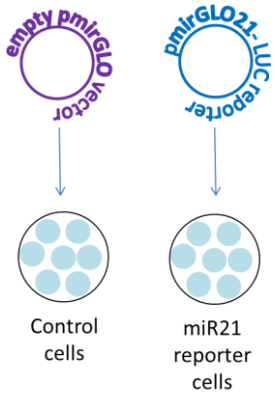
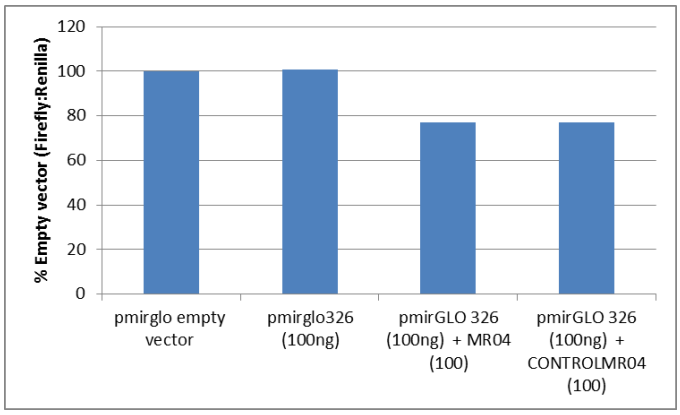


Figure 1. HeLa cells transfected with miR21 binding sequence
HeLa cells (7000 cells/well) were transfected with 200ng of pmirGLO empty vector or pmirGLO21 (containing mir21 binding sequence) using Lipofectamine. After 48 hours Dual Glo substrate was added to the cells and luciferase activity and Renilla Luciferase activity were measured. Results are calculated as ratio of Firefly:Renilla luminescence and normalized to the ratio from empty vector wells.

Next, we transiently transfected HeLa cells with the miR326 overexpression plasmid and the pmirGLO326 reporter vector. However, using this approach we did not see any alteration in reporter expression after miR326-MR04 addition compared to control MRO4 vector (Figure 2).



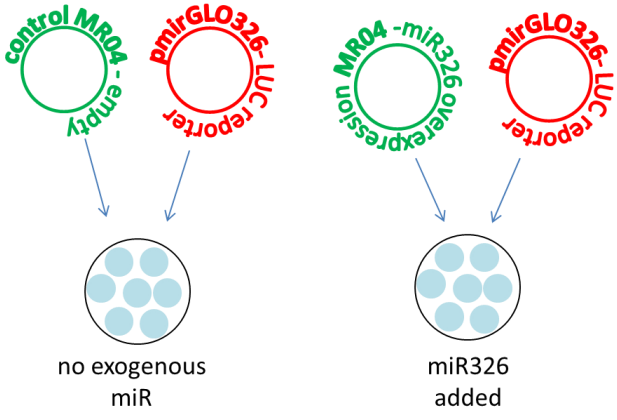


Figure 2. HeLa cell line transfected with miR326 binding sequence and miR326 or miR empty vector (MR04 control)
HeLa cells (7000 cells/well) were transfected with 100ng of pmirGLO empty vector or pmirGLO 326 (containing mir-326 binding sequence) using Lipofectamine. The two right columns received 100ng MR04 with miR326 overexpression or empty vector as well (as shown in the diagram on the right). After 48 hours Dual Glo substrate was added to the cells and luciferase activity and Renilla Luciferase activity were measured. Results are calculated as ratio of Firefly:Renilla luminescence and normalized to the ratio from empty vector wells.

We repeated the same transient transfection using the MCF-7 cell line that has been reported to express miR326 as well as miR21[6]. Again, the pmirGLO21 vector had little luciferase activity compared with empty vector. However, we did not detect a loss of luciferase activity

when pmirGLO326 was transfected, suggesting that the amount of endogenous miR326 was not sufficient to inhibit translation of the reporter construct. To overcome this, we co-transfected the pmirGLO plasmid with pMR04, a plasmid encoding miR-326, but even using this approach we did not observe any detectable reduction in luciferase activity (Figure 3). Sequencing of the pmirGLO326 plasmid showed that the construct had the appropriate binding site. Since the pMR04 plasmid also encodes GFP, we could determine the frequency of cells that were transfected with that plasmid by using flow cytometry. Very few cells expressed the GFP plasmid, and by extension, little miR326.

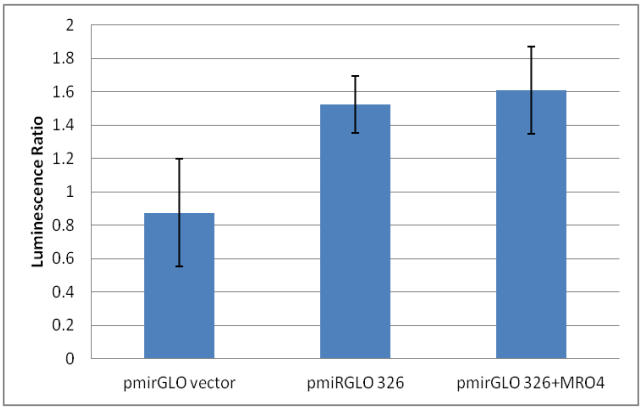


Figure 3. MCF-7 cell line transfected with miR326 binding sequence and miR326
MCF-7 cells (20000 cells/well) were transfected with 200ng of pmirGLO empty vector, pmirGLO 326, or pmirGLO326 and MR04 (encoding mir-326 overexpression) using Lipofectamine . After 48 hours Dual Glo substrate was added to the cells and Firefly and Renilla Luciferase activities were measured. Results are calculated as ratio of firefly:Renilla luminescence.

Since co-transfections with the MR04 plasmid were not successful, we then co-transfected the cells with a miR326 mimic. This is a synthetic RNA encoding the precursor form of miR326. Again, there was no evidence that the mimic was able to significantly suppress luciferase activity.

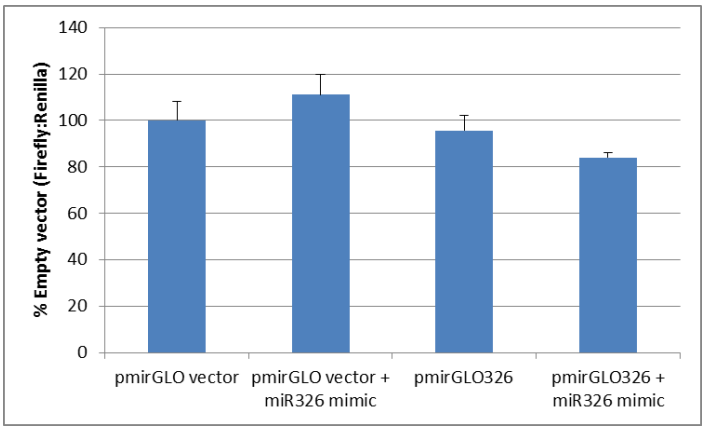


Figure 4. MCF-7 cell line transfected with miR326 binding sequence and miR326
MCF-7 cells (20000 cells/well) were transfected with 200ng of pmirGLO empty vector or pmirGLO 326 and miR326 mimic (90nM) using Lipofectamine . After 48 hours Dual Glo substrate was added to the cells and Firefly and Renilla Luciferase activities were measured. Results are calculated as ratio of firefly:Renilla luminescence.

We next created a stably transfected HeLa cell line that expresses miR326. The MR04 plasmid encodes a puromycin selection marker to facilitate generation of stable cell lines. Since puromycin has a very sharp kill curve, we performed a titration of puromycin to identify the optimal amount to use in our studies (0.5 ug/ml). Initially the stable transfections had shown some success as we observed GFP+ cells under the microscope 2 weeks following transfection (when the cells were growing in puromycin containing media). However, after another 2 weeks in culture (in the presence of puromycin) the transfected cells were GFP signal when observed

under the microscope. These cells also did not show any decrease in pmirGLO326 luciferase expression, indicating the stable transfections were not successful long-term.

We also attempted to generate a stably transfected HeLa cell line expressing pmirGLO326. The pmirGLO plasmid contains a neomycin resistance gene that allows for selection in G418 sulfate containing media. While the cells grew in G418 containing selection media, the expression of the control luciferase product (Renilla) was detected at very low levels. All subsequent attempts to increase the expression of the control Renilla reporter were not successful.

We next used lentivirus to create a stably transduced HeLa cell line that express miR326. The MR04 plasmid encodes a puromycin selection marker to facilitate establishment of stable cell lines. We grew the stably transfected cells in puromycin containing media, but there was very little reduction in pmirGLO326 luciferase signal compared to control pmirGLO transfected cells.

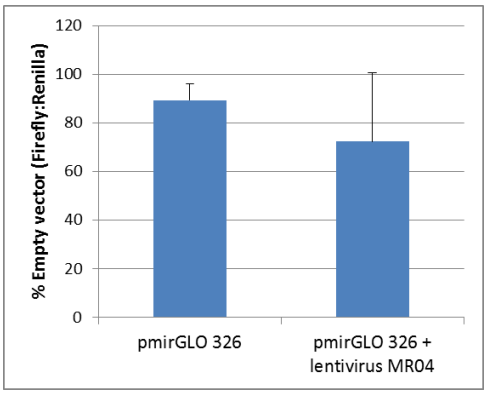


Figure 5. HeLa cell line transduced with lentivirus for miR326 (MR04), then transiently transfected with pmirGLO326 reporter

HeLa cells were transduced with MR04 lentivirus for miR326 overexpression (right column). Control pmirGLO326 was no transduced (left column). Both groups were transiently transfected with 200ng of pmirGLO 326. After 48 hours Dual Glo substrate was added to the cells and Firefly and Renilla Luciferase activities were measured. Results are calculated as ratio of firefly:Renilla luminescence compared to pmirGLO empty vector control.

While the efforts to create stable and transient transfectants were ongoing, we also set to replicate previously published data showing that small molecules are capable of inhibiting miR21 [7]. Cells transfected with the pmirGLO21 plasmid were treated with 2 compounds previously shown to inhibit the activity of miR21. In our hands, the addition of both compounds resulted in a slight increase of pmirGLO21 signal (note y-axis scale), indicating modest inhibition of miR21.

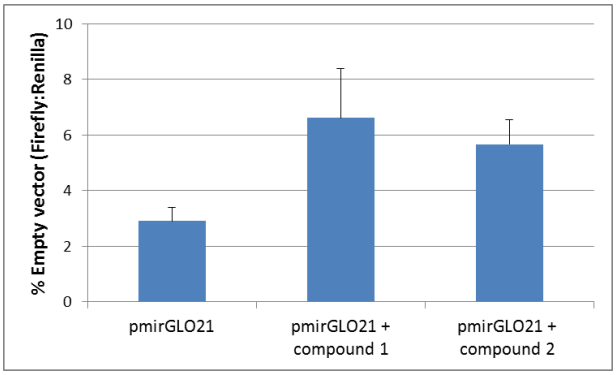


Figure 6. HeLa cell line transfected with pmirGLO21 assessed for inhibition with 2 previously published compounds.

HeLa cells were transiently transfected with pmirGLO21 and incubated in the presence of compound or media control. All groups were transiently transfected with 200ng of pmirGLO21. After 48 hours Dual Glo substrate was added to the cells and Firefly and Renilla Luciferase activities were measured. Results are calculated as ratio of firefly:Renilla luminescence compared to pmirGLO empty vector control.

We also used locked nucleic acid (LNA) sequences to inhibit any miR326 signal that was present to determine if we could perturb the pmirGLO326 signal. Treatment with miR326 LNA

increased the signal in the miR326/MR04 transiently transfected cells, but also the control

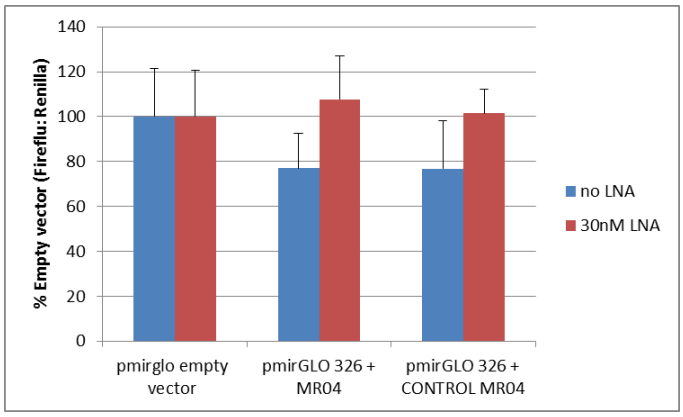
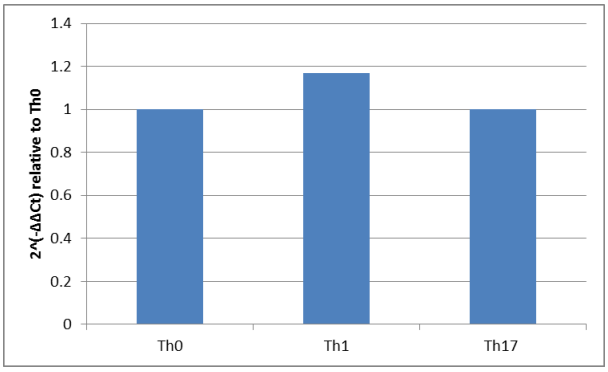


Figure 7. HeLa cell line transfected with pmirGLO326 and MR04 (overexpression miR326 or control) aassessed for inhibition with LNA specific for miR326.
HeLa cells were transfected with 100ng of pmirGLO empty vector or pmirGLO 326 and MR04 with or without 30nM LNA for miR326. After 48 hours Dual Glo substrate was added to the cells and luciferase activity and Renilla Luciferase activity were measured. Results are calculated as ratio of Firefly:Renilla luminescence and normalized to the ratio from empty vector wells.

MR04 plasmid.

Finally, we determined whether miR326 was detectable in T cells skewed to the Th17 lineage over the Th0 or Th1 lineage as shown previously [2]. Following the published skewing conditions [2], we achieved 35% IL-17+ cells in the Th17 conditions and 50% IFN-g+ cells in the Th1 conditions. MiRs were isolated from each cell culture (Th0, Th1, or Th17) using MirVana miRNA isolation kit (Life Technologies), then miR326 and U6 (housekeeping gene) transcripts were reverse transcribed (TaqMan MicroRNA Reverse Transcription Kit). Lastly, qPCR was performed and analyzed (Roche LightCyler480). The housekeeping gene Ct values were approximately 24 for all cell types and miR326 Ct was approximately 31.5 for all groups. Unlike previously published data, our RT-PCR analysis did not reveal any significant differences in miR326 expression across the various T cell groups.



While our efforts to develop a screening method for miR326 were not successful, others in the literature have since published varying results linking miR326 to MS. MiR326 has not been consistently detected in the blood or brain of MS patients and several microRNA screens in MS patients failed to detect significantly altered levels of mir326 [8] [9].

KEY RESEARCH ACCOMPLISHMENTS

- pmirGLO Dual-Luciferase miRNA Target Expression Vector: used as control. (Since there is no insert, the luciferase signal should be high).
- pmirGLO 326: has the insert for the miR-326 binding site. (If miR-326 is expressed, luciferase should be reduced).
- pmirGLO 21: has the insert for the miR-21 binding site, to be used as control miRNA during compound screening. (If inhibitor affects miR-21, luciferase should be increased).
- Tested overexpression of MR04-miR326 overexpression plasmid and empty MR04 plasmid for pmiRGLO326 reporter changes.
- Tested miR326 mimic for pmirGLO326 reporter changes.
- Developed LV-MR04-miR326 transduced HeLa cell line.
- Assessed endogenous miR326 in MCF7 cells for pmirGLO reporter changes.
- Determined no significant differences in mouse T helper cell expression of miR326 between naïve (Th0), Th1, and Th17 cells.
- Assessed the miR21 capabilities of previously published small molecule inhibitors of miR21.

REPORTABLE OUTCOMES

We developed plasmids that contain specific inserts as described in Key Research Accomplishments. We developed a miR326 overexpressing HeLa cell line. We measured no significant differences in mouse T helper cell expression of miR326. We measured minimal inhibition of miR21 by previously published compounds (compound 1 and 2).

CONCLUSION

To introduce miR326 into the cells, we selected the plasmid pEZX-MR04 vector (GeneCopoeia) encoding the precursor miRNA for human miR326. We also used lentiviral based vectors that are more resistant to epigenetic silencing to overexpress miR326 and did not observe significant differences in pmirGLO326 reporter expression. We measured T helper cell expression of miR326 and did not observe any significant increase for Th17 cells. We also determined the inhibition via previously published miR21 SMIs to be minimal for endogenous HeLa miR21. Transient transfection of pmiRGLO reporters was successful and it appears the challenges lie in reliable, exogenous overexpression of miR326.

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APPENDICES

None

SUPPORTING DATA

None